(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29225 A1

(51) International Patent Classification[7]: C12N 15/12, 15/10, 15/62, C07K 14/435, 14/47, A61K 38/02

(21) International Application Number: PCT/US00/08477

(22) International Filing Date: 29 March 2000 (29.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/160,461 21 October 1999 (21.10.1999) US
09/510,097 22 February 2000 (22.02.2000) US

(71) Applicant: PANORAMA RESEARCH, INC. [US/US]; Panorama Research, Inc., 2462 Wyandotte Street, Mountain View, CA 94043 (US).

(72) Inventor: BALINT, Robert, F.; Panorama Research, Inc., 2462 Wyandotte Street, Mountain View, CA 94043 (US).

(74) Agent: RAE-VENTER, Barbara; Rae-Venter Law Group, P.C., P.O. Box 60039, Palo Alto, CA 94306-0039 (US).

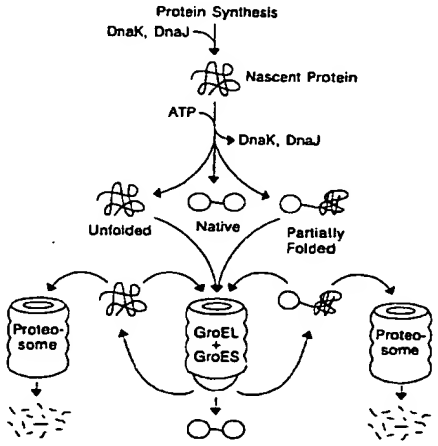
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A GENERAL METHOD FOR OPTIMIZING THE EXPRESSION OF HETEROLOGOUS PROTEINS



(57) Abstract: Methods are disclosed whereby variants of proteins which do not confer selectable phenotypes can nevertheless be selected for stable expression in heterologous hosts. Related methods are disclosed whereby cDNA expression libraries can be enriched for stable expression of autonomously folding domains in heterologous hosts. Related methods are further disclosed whereby peptides which stabilize unstable proteins may be selected from random peptide libraries. If a heterologous protein is expressed as a fusion with a selectable phenotype, the strength of the phenotype is proportional to the folding rate, and therefore the solubility of the protein of interest. Thus, the selectable phenotype can be used to select better expressors from libraries of mutagenized proteins of interest, or it can be used to select autonomously folding domains (AFD) from cDNA expression libraries, or it can be used to select peptides which stabilize unstable proteins.

WO 01/29225 A1

A GENERAL METHOD FOR OPTIMIZING THE EXPRESSION OF HETEROLOGOUS PROTEINS

Technical Field

This invention is related to methods and compositions for obtaining stable expression of a protein of interest, to obtain mutant proteins having enhanced stability as compared to wild type proteins, and to stabilize unstable proteins associated with disease by expressing the protein of interest as a chimeric protein composed of a selection marker and the protein of interest under selective conditions. The invention is examplified by preparation of highly fluorescent GFP mutants by expressing the GFP as a C-terminal fusion with chloramphenicol acetyltransferase.

INTRODUCTION

Background

Natural proteins have three fundamental properties in vivo which can be exploited to obtain stable expression of heterologous proteins in the absence of any distinguishing phenotype of the protein itself.

1. Natural proteins have unique minimum energy conformations.

In its broadest sense, a fold is simply a minimum energy conformation or ground state. The vast majority of sequences in protein sequence space do not have unique folds, but rather have multiple, inter-convertible minimum energy conformations (Li *et al.*, *Science* (1996) 273:666-669; Godzik, *TIBTECH* (1997) 15:147-151; Sauer, *Folding and Design* (1996) 1:R27-R30; Govindarajan and Goldstein, *Proc. Natl. Acad. Sci USA* (1996) 93:3341-3345). However, protein function is so exquisitely dependent on specific tertiary structure that it is generally not possible for a protein to be functional in more than one fold. For this reason evolution has selected sequences which fold cooperatively in the intracellular milieu into unique minimum energy conformations with high energy barriers between them and all kinetically accessible alternatives (Govindarajan and Goldstein, *Proc. Natl. Acad. Sci USA* (1996) 93:3341-3345). A general feature of these folds is a compact hydrophobic core. If, under adverse conditions of temperature, pH, ionic strength, etc., such as might occur during

physical or metabolic stress, the hydrophobic cores are disrupted or fail to form properly or in a timely fashion inside the cell, the exposed hydrophobic surfaces have a strong tendency to initiate intermolecular aggregation, which is generally toxic to cells. In fact, the aggregation of misfolded proteins has become increasingly recognized as a common etiologic component of disease (Wetzel, *Cell* (1996) 86:699-702). For this and other regulatory reasons cells have evolved highly efficient proteolytic mechanisms to detect exposed hydrophobic structure and prevent misfolded proteins from accumulating (Weissman *et al.*, *Science* (1995) 268:523-524; Coux *et al.*, *Ann. Rev. Biochem.* (1996) 65:801; Tamura *et al.*, *Science* (1996) 274:1385-1389; Löwe *et al.*, *Science* (1996) 268:533-539).

Nascent proteins partition among three fates in vivo: (1) folding into the soluble, functional, native ground state, (2) amorphous aggregation into insoluble inclusions, (3) proteolysis. Fate (1) is generally proportional to the folding rate. The faster a protein folds the more of it will reach the native ground state before succumbing to fates (2) and (3), which are essentially irreversible. With respect to stability, the most important effect of folding is to sequester hydrophobic surface, since exposed hydrophobic surface is the driving force for both aggregation and proteolysis. From these properties of natural proteins in vivo, it may be inferred that most natural proteins can only be stable in vivo in their native conformations in which they are also likely to be functional. Thus, most mutations which stabilize a natural protein in a heterologous host, will generally favor the native conformation, and will restore at least some measure of native functionality.

2. A multi-domain protein is only as stable in vivo as its least stable domain.

Inside the cell, nascent proteins first encounter the hsp40 and hsp70 classes of chaperone proteins and their associates which bind to any exposed hydrophobic sequence to protect the nascent protein in its unfolded state (Hartl, *Nature* (1996) 381:571-580). The new protein is then released from the chaperones by a cooperative, energy-dependant mechanism. Many proteins then fold with two-state kinetics, collapsing rapidly into their native folds without discernable intermediates. The remainder, however, may accumulate as 'molten globule' intermediates while searching for conformation space for their native folds. Since in this state they are vulnerable to aggregation or proteolysis, the hsp60 chaperonin system has evolved to provide a protective environment for slower folders (Hartl, 1996). Misfolded

proteins are drawn into the multimeric Hsp60 cylindrical complexes (illustrated in Figure 1), where they are bound to the inner surface in a fully extended state (Zahn *et al.*, *Nature* (1994) 368:261-265; Buckle *et al.*, *Proc. Natl. Acad. Sci. USA* (1997) 94:3571-3575). Each protein then undergoes cooperative, energy-dependant release into the cavity of the complex where it can attempt to fold without risk of aggregation or proteolysis. Thus, the folding machinery acts not by catalyzing or accelerating folding, but by protecting nascent proteins from alternative fates.

Any protein, new or old, which undergoes sufficient transient thermal or chemical denaturation to expose hydrophobic surface may be bound and unfolded by the chaperonin complex. Each protein may then undergo multiple rounds of binding, unfolding, release, and refolding until its native fold is achieved. However, after each round in which a protein still fails to achieve its native fold, it may either be rebound by the folding complex, or it may be bound by the protein turnover machinery, which also recognizes exposed hydrophobic surfaces. These alternative fates for nascent proteins are illustrated in Figure 1. Thus, the longer it takes a protein to fold, the more vulnerable it is to proteolysis or aggregation. Proteins which incur significant delays in folding in heterologous hosts may fail to accumulate for this reason.

There are many apparent parallels between mechanisms of protein folding and turnover in cells (Weissman *et al.*, *Science* (1995) 268:523-524). In both cases exposed hydrophobic surfaces are recognized and bound cooperatively, leading to unfolding of the entire protein within multi-subunit complexes having similar cylindrical architectures (Weissman *et al.*, *Science* (1995) 268:523-524; Zahn *et al.*, *Nature* (1994) 368:261-265; Buckle *et al.*, *Proc. Natl. Acad. Sci. USA* (1997) 94:3571-3575). However, whereas in the folding process the bound protein is released to fold again, in the proteosome, the bound protein is proteolyzed (see Fig. 1). Thus, the ability of proteins to accumulate in cells depends on both the rate of folding and the stability of the final fold, though recent work has suggested that these two may in fact be related (Scalley and Baker, *Proc. Natl. Acad. Sci. USA* (1997) 94:10636-40). From the foregoing it may be surmised that if any single domain in a multi-domain protein is unstable, the entire protein may be subject to proteosomal proteolysis. This is logical given that any surviving stable fragments from proteolysis of natural proteins would likely be either useless or deleterious, especially if they retained unregulated activity. Indeed, stable

fragments of natural proteins are rarely detected in cells unless they have functional significance. From this it follows that the strength of a selectable cellular phenotype should be proportional to the "foldability" of any domain to which the phenotype-conferring domain is fused, and that this proportionality could have many useful applications in protein engineering.

3. Mutations which stabilize proteins in vivo promote the native fold and function.

As discussed above, the vast majority of sequences in protein sequence space are not "foldable", i.e., they do not specify unique minimum energy conformations. The tiny fraction that do specify unique stable folds have been selected by evolution to serve as scaffolds for protein function. Interestingly, computational experiments have suggested that the most stable folds are also the most "designable" (Li *et al.*, *Science* (1996) 273:666-669). That is, they may be specified by the largest number of different sequences. This makes them evolutionarily stable as well as thermodynamically stable. Among natural proteins a number of "superfolds" have been observed, such as the immunoglobulin fold, a ~12 kDa sandwich of two 3-5-strand β-sheets, which has been adapted repeatedly to many different functions, specified by many different sequences (Padlan, *Molecular Immunology* (1994) 31:169-217). An increasing number of other studies have shown that extensive sequence alterations may be made in the cores of model proteins without substantially altering the native fold or function (Axe *et al.*, *Proc. Natl. Acad. Sci. USA* (1996) 93:5590-5594; Sauer, *Folding and Design* (1996) 1:R27-R30).

Many of the known protein folds have been observed in both prokaryotic and eukaryotic proteins (Netzer and Hartl, *Nature* (1997) 388:343-349). This is consistent with the fact that the intracellular milieus of prokaryotic and eukaryotic cells are quite similar with respect to bulk properties such as pH, ionic strength, protein concentration, etc. In spite of this, many natural proteins are unstable in heterologous hosts in that they either fail to accumulate to detectable levels, or when over-expressed they accumulate only as insoluble aggregates. Protein synthesis is ten times faster in prokaryotes than in eukaryotes (15 sec vs 2-3 min for a 40 kDa protein; Netzer and Hartl, *Nature* (1997) 388:343-349). This parallels cell division rates and allows each domain of a multi-domain protein to fold as it's made in eukaryotes, free from interference by simultaneously folding downstream domains. This adaptation accommodates the rise of multi-domain proteins in eukaryotes, which facilitate

compartmentalization of complex metabolic networks. In prokaryotes, protein synthesis is so fast that multiple domains are synthesized before they have time to fold, and may therefore interfere with the folding of each other. For this reason, prokaryotes have fewer multi-domain proteins, and many examples exist of prokaryotic single-domain proteins the eukaryotic homologs of which are linked into continuous polypeptides. Thus, inter-domain interference during folding may be a major factor contributing to the high failure rate for heterologous expression of eukaryotic multi-domain proteins in prokaryotes.

If most natural proteins have highly "designable" folds, then for each such protein there should be many sequences capable of specifying efficient, stable folding of the functional protein in heterologous environments. Natural proteins cannot be expected to fold any more efficiently than necessary in their natural milieus. In general, eukaryotic proteins may fold more slowly than prokaryotic proteins because the risk of aggregation is much greater in the prokaryotic cytoplasm. Because of the ten-fold higher prokaryotic protein synthesis rate, local concentrations of nascent proteins are much higher and nascent proteins have little chance to fold while still tethered to the ribosome. Folding is essentially a uni-molecular reaction and therefore independent of concentration, whereas, aggregation is effectively bi-molecular, and is therefore strongly favored by high concentrations of the protein in question. Since the insoluble fraction does not turn over, it grows monotonically, providing an ever increasing substrate for aggregation. As a result, the aggregation rate may rise exponentially, rapidly reaching a point where little nascent protein escapes. Thus, aggregation is a threshold-like phenomenon which

is exquisitely sensitive to small changes in parameters such as the folding rate and synthesis rate, which affect the initial aggregation kinetics.

The sampling of conformation space to find associations which nucleate cooperative assembly of the final fold is generally the rate-limiting step in folding, and the rate of this process is sharply limited by the energies of off-pathway interactions. Mutations which cause even modest destabilization of off-pathway interactions may accelerate folding sufficiently to increase soluble protein by several orders of magnitude. Thus, single mutations have been observed to accelerate folding up to 1000-fold, with double and triple mutants having even larger effects (Jackson, *Folding and Design* (1998) 3:R81-R91). For unstable heterologous proteins with selectable phenotypes, it should be possible to access such mutations by

combinatorial mutagenesis, selecting for restoration of the phenotype. For proteins which do not have selectable phenotypes, there is currently no reliable method to select for mutations which accelerate folding in a heterologous host. However, our demonstration of a tight correlation between the folding rate of proteins of interest and the strength of an artificially-linked phenotype, now makes it possible to identify such mutations in proteins of interest which lack selectable phenotypes. Often, over-expression of a misfolding protein as a fusion with an otherwise stable protein will improve the soluble yield of the misfolder. However, this works best when the stable partner is N-terminal, and has a chance to fold before the misfolder can aggregate or turn over. When the stable partner is C-terminal, as in our system, aggregation or proteolysis of the misfolder can commence before the stable partner can fold. In bacteria, the N-terminal stable partner may sterically hinder aggregation of the misfolding partner, and the local concentration of nascent misfolder is reduced by a factor approximately equal to 1 minus its proportion of the total molecular weight. Nevertheless, the strength of the selectable phenotype is still proportional to the solubility of the fusion protein, which in turn is limited by the aggregation rate of the slowest folding component.

We have shown that after random mutagenesis of a protein of interest using a low mutational operator, such as error-prone PCR (Cadwell and Joyce, in PCR Primer A Laboratory Manual, Dieffenbach and Dveksler (eds.) (1995) Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 583-590), DNA shuffling (Crameri *et al.*, *Nature Biotechnology* (1996) 14:315-9), random-priming recombination (RPR), (Shao *et al.*, *Nucleic Acids Research* (1998) 26:681-3), or the staggered extension process (StEP), (Zhao *et al.*, *Nature Biotechnology* (1998) 16:258-261) faster-folding variants can be selected by screening for higher levels of the selectable phenotype. Furthermore, as variants are selected which fold faster than the marker, the marker folding rate becomes limiting because even stable proteins will aggregate to some extent when over-expressed. However, as explained above, the marker is generally less prone to aggregation as the fusion than alone, so the maximum phenotype level produced by fusion of the marker with fast-folding variants may be even higher than that produced by the marker alone. Since the solubility of the fusion protein may become limited by the folding rate of the marker domain, the solubility of the optimized protein of interest may be even higher when expressed alone, without the marker fusion domain. Also, we have found that substantial improvements in expression can be achieved with single mutations, even

for proteins which already express well. We attribute this to the fact that rate-limiting intermediates may be readily destabilized by single mutations. This means that in mutagenic libraries with mutation frequencies on the order of one per molecule, the frequency of faster folders may be greater than ~one-tenth of the inverse of the chain length, or ~one in 2500 for a 250-residue protein. Thus, large libraries are not needed to find high-expressing variants of poor expressors. The fact that folding can be optimized with few mutations also minimizes the likelihood of introducing immunogenic epitopes into therapeutic proteins.

Optimization of bacterial expression of pharmaceutical/industrial proteins.

The industrial and pharmaceutical utility of many proteins is limited by prohibitive production costs, due to the difficulty of producing stable, functional protein in quantity. Most such proteins are not abundant either in their native sources, or in heterologous hosts. Combinatorial optimization of the expression of most such proteins has previously been limited to those which confer selectable phenotypes on the production host. However, with the subject invention it is now possible to optimize the expression of any protein in any heterologous host by mutagenizing the protein and expressing the mutant library as a C-terminal fusion with a selectable marker. Mutations which accelerate folding are selected on the basis of the strength of the marker phenotype. Selected clones should be enriched for native activity such that only a modest number will have to be screened to recover the desired activity. The benefits of optimized expression are manifold. Not only are yields increased and production and purification costs lowered, but higher levels of purity are often possible when the desired product is a higher proportion of the starting material.

Since proteins have evolved to fold only as efficiently as necessary in their native environments, it is reasonable to expect that most proteins could be mutated to fold more efficiently. The absolute limits of folding efficiency in vivo are not known, but with the subject invention, it may be possible to test those limits. First, selectable markers can be optimized by mutagenesis and selection for maximum strength of phenotype. If such folding-optimized markers have any remaining tendency to aggregate when over-expressed, it will be even further reduced when they are expressed as fusions to mutagenized proteins of interest. Thus, folding-optimized markers should place no limit on the optimization of proteins of interest.

This could allow valuable proteins to be produced in higher yields with higher activities and purity than previously possible. Also, fast-folding variants are likely to be more thermodynamically stable as well, since recent experimental (Plaxco *et al.*, *J. Mol. Biol.* (1997) 270:763-70; Mines *et al.*, *Chem. Biol.* (1996) 3:491-7) and theoretical (Gutin *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:1282-6; Wolynes *et al.*, *Chem. Biol.* (1996) 3:425-32) work suggests that folding rates are closely correlated with stability of the native state. This is not unexpected if mutations which accelerate folding by destabilizing off-pathway intermediates also stabilize the native conformation by reducing the ensemble of kinetically-accessible alternatives. Thus, it will be important to compare the thermal stabilities of fast-folding variants and their wild-type precursors.

There are two types of stability in proteins. The first relates to tolerance of extreme conditions, and the second relates to half-life under favorable conditions. They are not necessarily mutually inclusive. The reason for this is that activity is often lost reversibly before it is lost irreversibly, but the reverse is not possible. In fact, if loss of activity under extreme conditions were entirely reversible, it would have little to do with the half-life of the protein, which is primarily a function of the rate of irreversible aggregation. Each trait is potentially valuable for industrial proteins. Proteins which work better under extreme conditions, and/or last longer will fetch a premium on the market, in addition to savings realized from reduced production costs, and possible premiums for higher purity. Folding optimization selects primarily for reduced tendency to aggregate. Since aggregation is the principal route of irreversible inactivation, this should prolong the half-life. So long as this is accomplished by destabilizing aggregation-prone intermediates without undue effect on the enthalpy or entropy of the ground state, reversible stability should not be adversely affected.

Efficient searching of protein libraries in vivo will require pre-selection for stability.

Current efforts to accelerate the discovery and validation of new therapeutic and diagnostic targets and reagents to meet growing health care and pharmaceutical industry demands depend heavily on continuing development of new and improved recombinant DNA-based protein engineering methods. For example, to realize the potential of genomics for the identification of new therapeutic targets, high-throughput methods for the functional analysis of expressed sequences of interest will be required. One important approach to rapid

functional analysis will be to use protein-protein interaction traps to identify networks of interactions within and between the proteomes of human cells, tissues, and pathogenic organisms, using cDNA expression libraries. However, current methods for the construction of cDNA libraries for fusion expression, as required for interaction trapping are so inefficient that recovery of only the most abundant and robust ligands can be expected. The vast majority of cDNA sequences in such libraries are not stably expressed, either because the reading frame is incorrect, or because the encoded fragment is not in register with a foldable domain.

Current recombinant DNA methods allow the construction of cDNA libraries in bacteria containing up to 10^9 independent clones, or $> 10^4$ times the average number of expressed genes in mammalian tissues. If the frequencies of the rarest genes are assumed to be in the 10^{-6} range, and on average only one in a hundred clones of a gene make a stable interaction-competent product, there would still be ten such clones for each rare expressed sequence in a library of 10^9. Thus, the initial size of the library is not theoretically limiting. What limits recovery from these libraries with respect to the expression host is the transformation efficiency, and with respect to the screen recovery is limited by throughput and signal-to-noise ratio. In yeast, libraries are limited by transformation efficiency to $\sim 10^6$-10^7 clones. In mammalian cells the limit is $\sim 10^5$-10^6. Thus, comprehensive searching of such libraries in eukaryotic hosts will require not only enrichment for stable expressors, but also normalization to bring the frequencies of rare expressors within range of the library size limits.

Throughput is limited by the ability to detect positive clones in the presence of an excess of negative clones. Clone-by-clone screens have the lowest throughput, color screens have intermediate throughputs, and viability screens generally have the highest throughputs. However, even when neither transformation nor screening is limiting, as with biopanning or viability selection in a bacterial expression host, recovery is still limited by the "needle-in-a-haystack" problem, whereby the discriminating power of the screen, or "signal-to-noise" ratio determines the minimum product of frequency and affinity which can be selectively enriched above background. Thus, if on average, only one randomly-primed cDNA fragment in a hundred expresses a stable, functional domain in the heterologous host (a conservative estimate), then the frequencies of all such clones could rise by up to two orders of magnitude if a way could be found to eliminate the non-expressors from the libraries before screening.

Such an enrichment could make the critical difference for recovery of many important interactors.

Relevent Literature

Albano *et al.*, *Biotechnol Prog* (1998) 14:351-4 describes the use of the correlation between the fluorescent intensity of the reporter GFP and the functional activity of a protein to which it is fused. Similarly, Waldo *et al.*, *Nature Biotechnology*, (199) 17:691-695, describes the use of GFP to predict solubility of a protein of interest by expressing it as a chimeric with the protein of interest. *See* also PCT/US98/25862.

SUMMARY

Methods are provided for obtaining host cells expressing a mutant of a desired protein optimized for expression in the host cells, for obtaining a protein with enhanced stability as compared to a wild type of the desired protein, and for identifying peptides that can stabilize an unstable protein, in each case by expressing the protein linked to a selector protein that confers a selectable phenotype on the host cell. In the method for identifying stabilizing peptides, the unstable protein is coexpressed with members of a random peptide library. To obtain optimized expression of a protein in a host cell, the method includes the steps of preparing a library of mutagenized coding sequences for the protein of interest, purifying the members of the library of mutagenized coding sequences, ligating each member of the library into an expression cassette in frame with the coding sequence for a selector protein, transforming a multiplicity of host cells with the expression cassettes, growing the resulting transformed host cells under conditions for which the selector protein confers ability for the transformed cells to grow to produce the mutant proteins joined to the selector protein, identifying cells that express mutant proteins at a selective pressure higher than that of cells expressing an unmutagenized protein. Proteins with enhanced stability can be obtained by cleavage from the selector protein, or expressing the mutant protein as a free protein in the host cells for which it is optimized. The invention finds use for example in optimizing mammalian peptides for improved expression in prokaryotic

cells and for identifying peptides that can be used for treating diseases that are characterized by production of an unstable variant of a wild type protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. An illustration of the alternative fates for nascent proteins when expressed in cells at normal levels. When overexpressed, aggregation is an additional fate (not shown). DnaK and DnaJ are bacterial Hsp70 and Hsp40 proteins, respectively. GroEL is the bacterial Hsp60 complex, and GroES is the companion Hsp10 complex. For 2-domain proteins like GFP-CAT, we hypothesize that misfolding of a single domain leads to turnover of the entire protein.

Figure 2. Expression construct for GFP-CAT fusions. T7prom, phage T7 promoter; $(G_4S)_3$, flexible spacer between the GFP and CAT domains; His_6, hexa-histidine tail for affinity purification; T7t, phage T7 transcription terminator; ori, origin of replication; bla, ampicillin resistance. Arrow denotes start of translation.

Figure 3. Chloramphenicol resistance of *E. coli* NovaBlue DE3 cells expressing CAT, wtGFP-CAT, and GFPuv-CAT. Cells expressing each construct were plated at 1000 cells per plate onto solid LB medium containing 0.02 mM IPTG and icreasing concentrations of chloramphenicol. After overnight growth at 37° C colonies per plate were scored and plotted against cam concentration.

Figure 4. Correlation of chloramphenicol resistance with fluorescence intensity in cells expressing mutagenized GFP as the GFP-CAT fusion. Mutant library transformants were seeded at 1000 per plate on increasing concentrations of cam. The percentage of colonies fluorescing brighter than wtGFP-CAT was determined visually and plotted against cam concentration.

Figure 5. Fluorescence emission spectra for cells expressing four GFP-CAT constructs. GFP-CAT expression was induced during log phase growth in suspension, after which the cells were washed and adjusted to a density of $O.D._{600} = 0.1$. Emission spectra were then taken at an excitation wavelength of 390 nm.

Figure 6. Selection of protein-stabilizing peptides from random peptide libraries (RPL) using the Fold Selector system. Figure A. Genes for unstable extra-cellular proteins of choice

(p.o.c.), such as amyloid β protein (Aβ), fused to the N-terminus of β-lactamase via a flexible linker, $(G_4S)_3$, may be transcribed from the *trp-lac* fusion promoter (*trc* prom) in a p15A replicon (p15A ori) with kanamycin resistance (*kan*) for plasmid retention. N-terminal signal peptides (SP) on this and the RPL fusion protein allow export of the gene products to the *E. coli* periplasm. The RPL genes, encoding random peptides fused to the N-terminus of thioredoxin via a G_4S linker, may be transcribed from the *lac* promoter in a pUC phagemid with chloramphenicol resistance (*cat*) for plasmid retention. The phagemid origin of replication (f1 ori) allows the RPL construct to be packaged in phage and quantitatively introduced into cells expressing the unstable protein by infection at high multiplicity (m.o.i.). Peptides are selected by their ability to stabilize the p.o.c. and thereby confer growth on non-permissive antibiotic concentrations. Figure B. Expression of unstable intra-cellular proteins is similar except that chloramphenicol acetyl transferase (CAT) is used as the C-terminal fusion to allow selection of stabilizing peptides for chloramphenicol resistance. Also, SPs are eliminated to allow retention of expressed proteins in the cytoplasm, and ampicillin resistance (*amp*) is used for p15A plasmid retention.

BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods for obtaining a protein of interest that is optimized for expression in a host cell, particularly a prokaryotic cell to which the protein of interest is heterologous are provided. To obtain a protein that is optimized for expression in a particular host cell, such as *E. coli*, members of a library of mutagenized coding sequences for the protein of interest joined to the coding sequence for a selector protein are transformed into the host cells which are then grown under conditions for which the selector protein confers ability for the transformed cells to grow. Generally the coding sequence for the chimeric protein contains a coding sequence for a linker peptide between the mutangenized library member and the coding sequence for the selector protein; prefereably the linker is a flexible linker. The selector protein can be any protein that provides for a selectable phenotype, such as antibiotic resistance. The protein of interest need not have a selectable phenotype. Cells that express the mutant proteins joined to the selector protein at a selective pressure that is nonpermissive for host cells expressing an unmutagenized protein are those that contain a protein optimized for expression in the host cell. The mutant proteins can be further screened to identify those

that have retained one or more wild type function, and also can be screened to identify those that have one or more altered characteristic, such as increased solubility, increased half life and decreased temperature sensitivity.

Also provided are methods for screening for peptides, particularly peptides of from about 3 to 20, generally of about 12 or less amino acids, that can stabilize unstable proteins such as those associated with particular disease states. A chimeric protein that includes the defective protein and a selector protein is coexpressed with a member of a tethered random peptide library in a host cell grown under selective conditions. The members of the library are each a linear chain of about 3 to 20 amino acids, preferably a linear chain of 12 or less amino acids, fused via a flexible linker to a stable carrier. Growth of cells under selective conditions is indicative of cells that contain a peptide which stabilizes the defective protein, which can then be identified and screened to assess whether it can also stabilize a free defective protein.

Description of the Invention

What is demonstrated is the feasibility of using a surrogate marker domain is demonstrated in a two-domain fusion with a protein of interest in *E. coli* to select mutagenic variants of the protein of interest with improved expression as a function of accelerated folding kinetics. We accomplished this goal by demonstrating a high degree of correlation between the functional stabilities of a selectable phenotype, chloramphenicol resistance, and a test protein, GFP, in *E. coli*. Chloramphenicol resistance is conferred by chloramphenicol acetyl transferase (CAT), which has been stably and functionally expressed as both N- and C-terminal fusions with many heterologous proteins (e.g., Dekeyzer *et al.*, *Protein Engineering* (1994) 7:125-130; Zelazny and Bibi, *Biochemistry* (1996) 35:10872-10878). Functional GFP and folding variants thereof (Stemmer, *Proc. Natl. Acad. Sci. USA* (1994b) 91:10747-10751) emit a bright green fluorescence in blue or uv light. The fluorescent chromophore of GFP is formed autocatalytically in the folded protein by cyclization of the peptide backbone of Ser65, Tyr66, and Gly67 (Cubitt *et al.*, *Trends inBiochem. Sci.* (1995) 20:448-455). GFP has also been stably and functionally expressed as both N- and C-terminal fusions with many heterologous proteins (Cubitt *et al.*, *Trends inBiochem. Sci.* (1995) 20:448-455).

Previously, we had attempted to isolate spectral variants of GFP from mutagenic libraries for use in a fluorescence energy transfer-based protein-protein interaction trap

(Delagrave *et al.*, *Bio/Technology* (1995) 13:151-154; Mitra *et al.*, *Gene* (1996) 173:13-17). In the course of that work, many non-fluorescent GFP variants were observed during mutant library screening. Western blot analyses revealed that for most of these variants, soluble, recombinant GFP protein failed to accumulate in cells under conditions in which soluble wild type GFP was readily detectable (Mitra and Balint, unpublished). However, in most cases full-length GFP protein could be recovered from the insoluble fraction of both mutant and wt GFP-expressing cells in comparable amounts. These observations suggested that fluorescence-null GFP variants arise from destabilizing structural or folding mutations more frequently than from active site mutations, i.e., mutations which inhibit chromophore formation but not folding. This is consistent with the fact that in most proteins, active sites are smaller mutational targets than structure-determining sites, and this can be estimated for GFP from the known x-ray crystal structures (Ormö *et al.*, *Science* (1996) 273:1392-1395; Yang *et al.*, *Nature Biotechnology* (1996) 14:1246-1251). The availability of x-ray structures and known folding mutations in a protein which is otherwise stably and functionally expressed in *E. coli*, make GFP an useful tool for testing the concept that the correlation of the stability of one domain with the activity of another domain in a multi-domain protein can be used to isolate stable variants of proteins which do not have selectable phenotypes. While the invention is exemplified with GFP as the protein to be stabilized, and neomycin as the selectable marker, any protein which is desired to stabilize can be substituted for GFP and any selectable marker can be substituted for neomycin. Likewise, while the invention has been exemplified in *E. coli*, any other host cell of interest either prokaryotic or eukaryotic, can be substituted for *E. coli*.

The following examples are offered by way of illustration of the present invention, not limitation.

EXAMPLES

Example 1

Correlation of functional GFP with chloramphenicol resistance for wild-type GFP and a fast-folding variant expressed as C-terminal fusions with CAT in *E. coli*.

The coding sequences for wild-type (wt) GFP and a highly-expressing variant of GFP (GFPuv), (Crameri *et al.*, *Nature Biotechnology* (1996) 14:315-9) were inserted into the pET23a vector (Novagen, Inc.) between NheI and BamHI (see Figure 2). pET23a is an ampicillin-resistant pBR322 derivative in which transcription of inserted coding sequences is controlled by the bacteriophage T7 promoter and transcription terminator (Moffatt and Studier, *J. Mol. Biol.* (1986) 189:113-130). Expression is restricted to hosts, such as NovaBlue (DE3) (Novagen, Inc.), which have been transformed to express the T7 RNA polymerase. GFP fluorescence can be readily observed in colonies of these cells harboring the pET-GFP construct by illuminating with long-wave uv light. The spectrum, quantum yield, and extinction coefficient of GFPuv do not differ appreciably from wtGFP, consistent with a difference of only three of 238 amino acids (Crameri *et al.*, *Nature Biotechnology* (1996) 14:315-9). However, when expressed from identical constructs in *E. coli* cells GFPuv produces 30-45 times more steady state fluorescence than does wtGFP. Since the specific fluorescence of both proteins is comparable, it may be concluded that the higher fluorescence intensity of cells expressing uvGFP is due to a comparable increment in the steady state amount of soluble, functional uvGFP protein. This is supported by data indicating that the total amount of GFP protein in the cells is comparable for both, but that proportionally more GFPuv is present in the soluble pool. Thus, the mutations in GFPuv appear to have increased its steady-state activity in *E. coli* by specifically reducing its tendency to aggregate, presumably as a result of an increased folding rate.

To use a surrogate marker to select for more stable variants of a protein of interest, the selectable marker coding sequence must be inserted downstream from that of the protein of interest to insure that selection is not favored by premature termination of the protein of interest. Thus, the CAT coding sequence was inserted into the XhoI site of pET23a in the same reading frame as the upstream GFPs. Between the two a 15-residue flexible, hydrophilic linker, $(Gly_4Ser)_3$, was encoded with convenient restriction sites for facile replacement of both

GFP and CAT sequences. The CAT sequence terminates in a His_6 tail for facile purification. This construct, pET23a-GFP-CAT is shown in Figure 2.

Table I and Figure 3 show the results of comparisons of the chloramphenicol resistance and fluorescence characteristics of wtGFP and GFPuv expressed alone and as C-terminal fusions with CAT from pET23a in *E. coli* strain BL21(DE3). When expressed alone, GFPuv produces ~30 times more steady state fluoresence than wtGFP, as determined by fluorometry of suspensions of equal numbers of cells from overnight growth on solid medium. Maximum transcription normally requires induction of T7 polymerase expression with IPTG, but a low level of transcription occurs even in the absence of IPTG. Interestingly, induction of wtGFP by IPTG produces no detectable increment in fluorescence over the uninduced level, though the level of wtGFP protein is considerably higher, as determined by gel electrophoresis. This suggests that at the uninduced expression level, aggregation is minimal, probably due to sub-threshold concentrations of nascent GFP. This is supported by the fact that under uninduced conditions, GFPuv fluorescence is comparable to that produced by wtGFP, which would be expected if wtGFP fluorescence is not limited by aggregation. At the induced expression level, however, substantial insoluble material forms in the wtGFP-expressing cells, presumably due to much higher nascent wtGFP concentrations. Under the same conditions GFPuv produces ~30-fold higher fluorescence intensity. From this we conclude that GFPuv is much less prone to aggregation at similar expression levels, i.e., nascent protein concentrations, presumably due to a higher folding rate.

Table I. Comparison of Relative Steady-State Fluorescence Intensity and Chloramphenicol Resistance (Cam^r) for CAT Fusions of wtGFP and GFPuv in *E. coli*.

Expression Product	IPTG	Cam^r [a]	Fluorescence [b]
wtGFP	0	NA	1x
	0.02 mM	NA	1x
GFPuv (3 mutations)	0	NA	1x
	0.02 mM	NA	30x
CAT	0	34 µg/ml	NA
	0.02 mM	306 µg/ml	NA
wtGFP-CAT	0	34 µg/ml	1x
	0.02 mM	238 µg/ml	2x
GFPuv-CAT	0	34 µg/ml	1x
	0.02 mM	340 µg/ml	4x

a. Chloramphenicol resistance (Cam^R) was determined as the highest concentration in solid LB medium on which at least 50% of cells plated formed visible colonies after overnight growth.

b. Fluorescence was determined by fluorometry at λexcite = 395 nm and λemit = 508 nm of cell suspensions of 0.1 OD600 after overnight growth on solid LB medium.

Interestingly, when induced, cells expressing CAT alone were resistant to less chloramphenicol than cells expressing GFPuv-CAT. This suggests that GFPuv, derived from a jellyfish protein adapted to life at ~ 13°C, is less prone to aggregation when over-expressed

in bacteria at 37°C than is CAT, a native bacterial protein. Consistent with this, cells expressing induced GFPuv-CAT are much less fluorescent than cells expressing GFPuv alone, suggesting that the stability of GFPuv-CAT is limited by the stability of CAT. The improvement in CAT expression in GFPuv-CAT is probably due to its reduced concentration in nascent protein (CAT is less than half the size of GFPuv-CAT and their synthesis rates should be similar), and/or GFPuv may sterically interfere with CAT aggregation. On the other hand, wtGFP appears predictably to be less stable than CAT, since induced wtGFP-CAT confers less chloramphenicol resistance than CAT alone, but more fluorescence than wtGFP alone. In general, SDS PAGE analyses of soluble and total extracts were consistent with the fluoresence and chloramphenicol resistance phenotypes, i.e., higher soluble protein levels correlated with higher fluorescence and higher cam resistance, though total protein remained more or less constant.

Example 2

Isolation of super-stable variants of GFP from mutagenic library expressed as fusion with CAT and selected for increased chloramphenicol resistance.

The GFP coding sequence may be subjected to random mutagenesis by any of several methods, including error-prone PCR (Cadwell and Joyce, in PCR Primer A Laboratory Manual, Dieffenbach and Dveksler (eds.) (1995) Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 583-590), DNA shuffling (1994a,b), random-priming recombination (RPR), (Shao *et al.*, *Nucleic Acids Research* (1998) 26:681-3), or the staggered extension process (StEP), (Zhao *et al.*, *Nature Biotechnology* (1998) 16:258-261). The wtGFP coding sequence may be amplified from pET-wtGFP using primers containing the NdeI site at the translation start, and at the unique EcoRI site just beyond the GFP C-terminus in pET-GFP-CAT. The error-prone amplification reaction is carried out in the presence of excess Mg^{++} and excess deoxynucleoside triphosphates to encourage mis-incorporation. An excess of primers may also be used, since the final mutation frequency is proportional to the number of cycles, so long as the primers are not exhausted. Under standard error-prone conditions (Cadwell and Joyce, in PCR Primer A Laboratory Manual, Dieffenbach and Dveksler (eds.) (1995) Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 583-590), a mutation frequency of ~0.7% is produced in the final product after 25-30 cycles. Since 75% of coding mutations produce

coding changes, we would expect ~3-4 amino acid substitutions per GFP clone from this protocol. The GFP coding sequence in plasmid pGFP (Clontech Laboratories, Inc.) was used as template for error-prone amplification using the end-specific primers AGCAGTCGCTTCACGTTCGCTCGC and GCATTCATCAGGCGGGCAAGAATG. The template GFP sequence was that reported by Chalfie *et al.* (1994) with the following changes: the starting triplet MGK has been replaced by MASK derived from the pET23a multiple cloning site, and the final SG has been replaced by NS. The same changes were also introduced into the GFPuv sequence (Clontech Laboratories, Inc.). Additionally, a Q80R mutation derived from a PCR error has been retained. The error-prone PCR product was gel-purified, and ligated back into the GFP-CAT fusion expression construct shown in Figure 2. The ligation product was then introduced into cells of *E. coli* strain NovaBlue (DE3) by high-voltage electroporation. Transformants were then plated onto solid Luria-Bertani medium containing increasing amounts of chloramphenicol, ranging from 34 μg/ml to 544 μg/ml, and incubated at 37°C overnight.

An initial assessment of the correlation of cam resistance with fluorescence intensity was made by a visual estimation of the percentage of colonies which fluoresced more intensely than wtGFP-CAT as a function of cam concentration. The results are illustrated in Figure 4. As the concentration of cam increased, the frequency of brighter colonies also increased. At cam concentrations above 270 μg/ml, the probability of visually identifying a brighter colony rose above 10%. When these brighter clones were restreaked, they invariably remained brighter than wtGFP-CAT-expressing cells. Interestingly, when colonies resistant to high cam were replated onto 34 μg/ml cam, the percentage of brighter colonies rose to ~30%. Thus, many clones expressing brighter GFP variants were masked by the high concentrations of cam used for selection which probably inhibited protein synthesis somewhat.

From the first round of mutagenesis colonies were recovered from cam concentrations of up to 408 μg/ml, exceeding the cam resistance of the GFPuv-CAT construct. For the purpose of phenotypic screening, ten clones were picked from plates containing between 306 and 408 μg/ml cam. These clones were selected solely for their ability to grow on cam concentrations which were non-permissive for the parental wtGFP-CAT construct. In ambient room light GFP fluorescence was not visible. For a second round of mutagenesis forty clones from the first round were pooled in ambient light from cam concentrations which were non-

permissive for wtGFP-CAT. Plasmid DNA was purified from the pooled clones and used as template for mutagenesis by the staggered extension process (StEP) for *in vitro* recombination of mutations selected from the first round (Zhao *et al.*, *Nature Biotechnology* (1998) 16:258-261). This method employs a template switch recombination mechanism, in which a short extension time is used to allow only partial replication of the sequence during each cycle. Thus, each full-length copy is generated over several cycles with the template being switched between each cycle. The second round product was ligated back into the vector as before and plated onto the same range of cam concentrations as used for the first round. Colonies were observed on cam concentrations of up to 510 μg/ml. Again, for phenotypic screening, 10 clones were picked in ambient room light from plates containing between 306 and 510 μg/ml cam.

The 20 total clones selected from cam plates in rounds one and two were grown in suspension in 5 ml of LB containing either 100 μg/ml ampicillin or 34 μg/ml chloramphenicol. At an $O.D_{600}$ of 0.4, protein expression was induced with 0.4 mM IPTG. After 16 hours of overnight growth, cells were washed and resuspended in phosphate buffered saline (PBS) at OD_{600} of 0.1, and the whole cell fluorescence was measured by excitation at 395 nm and emission at 508 nm. Fluorescence emission spectra were then determined for the brightest mutants from each round, designated GFPR1-CAT and GFPR2-CAT respectively. Each had an emission maximum at 510 nm when excited at 390 nm. The emission spectra of these clones are compared to those of wtGFP-CAT and GFPuv-CAT in Figure 5. To assess the effect of the CAT gene on GFP expression, stop codons were inserted at the C-termini of the coding sequences of GFPwt, GFPuv, GFPR1, and GFPR2 in the GFP-CAT fusion constructs to allow expression of the free GFPs. The relative fluorescence intensities for GFP expression with and without CAT are summarized in Table II.

Table II. Relative Fluorescence Intensities of GFP Constructs

CONSTRUCT	Relative Emission Intensity at 510 nm
GFPwt-CAT	1
GFPuv-CAT	4
GFPR1-CAT	14
GFPR2-CAT	56
GFPwt	1
GFPuv	30
GFPR1	40
GFPR2	40

The brightest mutant from round one, GFPR1-CAT, was 14 times brighter than wtGFP-CAT and 3.5 times brighter than GFPuv-CAT. GFPR1-CAT was also resistant to at least 408 μg/ml cam, whereas GFPuv-CAT could resist only 340 μg/ml. The brightest mutant from round two, GFPR2-CAT, was 56 times brighter than GFPwt and 14 times brighter than GFPuv-CAT, and could grow in 510 μg/ml cam. Interestingly, the dramatic increases in expression seen with GFPR1-CAT and with GFPR2-CAT over GFPuv-CAT essentially vanished when CAT was removed. All three expressed at comparable levels, 30-40 times that of wtGFP. Whereas GFPuv expression was inhibited by fusion to CAT by 7-8-fold, expression of GFPR1 was inhibited only ~3-fold, and expression of GFPR2 was actually enhanced slightly by fusion to CAT. Thus, not only were mutations selected which enhanced expression of the free GFP protein, but mutations were also selected which specifically

enhanced expression of GFP as fusions with other proteins. The reduced expression of GFPuv-CAT relative to free GFPuv is probably not due to dominant weaker expression of CAT because CAT does not have the same effect on GFPR2. Rather the reduced expression of GFPuv-CAT is probably due to mutual steric interference with the folding of the two proteins, and the same is probably true to a lesser extent for GFPR1-CAT. SDS-PAGE confirmed that the GFPR1-CAT, GFPR2-CAT, GFPwt-CAT, and GFPuv-CAT all comprised over 50% of the total cell protein. The increase in brightness for the GFPR1-CAT and GFPR2-CAT mutants over that of wtGFP-CAT and GFPuv-CAT is reflected by the difference in the amount of protein in the soluble fraction. For example, about 25% of GFPR2-CAT protein is soluble, whereas only about 1-2% of wtGFP-CAT protein was soluble.

DNA sequences were determined for the entire open reading frames of GFPR1 and GFPR2, and compared to those of wtGFP and GFPuv (see Table III). In addition, the reported mutations in GFPuv were confirmed. Surprisingly, one mutation was shared by all three improved GFPs, V164A. Even more surprisingly, this was the only mutation present in GFPR1. Since GFPR1 expresses as well or better than GFPuv as the free protein, this suggests the other mutations in GFPuv are not necessary. GFPuv had originally been "evolved" by repeated rounds of recombinatorial mutagenesis by DNA shuffling and phenotypic selection, followed by back-crossing to eliminate deleterious mutations (Crameri *et al.*, *Nature Biotechnology* (1996) 14:315-9). However, it appears that only one of the three remaining mutations is actually required for the complete phenotype. Thus, not only was recombination unnecessary, but the required mutation could have been recovered easily from a few thousand clones of a standard *Taq* polymerase amplification of the wtGFP coding sequence. Under standard conditions ~25 cycles of PCR with a non-proofreading polymerase such as *Taq* produces one mutation per ~700 bp, which is roughly the size of the GFP coding sequence. Since only a single nucleotide change with a frequency of 1/3 is required for the V164A mutation, the expected frequency would be one in only ~2100 clones.

Table III. Sequence Comparison of Three High-Expressing GFP Variants and wtGFP

Amino Acid Residue	GFPwt	GFPuv	GFPR1	GFPR2
100	TTT (F)	TCT (S)	TTT (F)	TTT (F)
105	AAC (N)	AAC (N)	AAC (N)	AGC (S)
154	ATG (M)	ACG (T)	ATG (M)	ATG (M)
164	GTT (V)	GCT (A)	GCT (A)	GCT (A)

Since the V164A mutation could account for all of the increase in free GFP expression for all three improved GFPs, we wished to see if any other independently adaptive mutations could be recovered. Before embarking on the arduous task of sequencing a large number of additional clones, however, we first examined the eighteen other independent clones selected from rounds one and two, which grew on non-permissive cam, for the presence of the V164A mutation. This mutation was present in all eighteen clones. Thus, V164A appeared to be the only single-hit mutation capable of destabilizing the aggregation-prone intermediate in GFP folding. Indeed, such a mutation would be expected to reduce the hydrophobicity at that position, and it is hydrophobicity which would be expected to drive aggregation. Any other independently adaptive mutation of comparable frequency should have appeared at least once. Of course, it is possible, even likely, that combinations of two or more mutations could have had a comparable effect, but their frequency would have been too low to be readily selected from our library.

Neither of the other two mutations present in GFPuv appeared in any of the twenty selected clones, consistent with their apparent dispensibility for increased cam resistance. More revealing, however, is the fact that GFPR1 showed 3.5-fold higher expression as the

CAT fusion than GFPuv-CAT. This suggests that at least one of the two other mutations in GFPuv is responsible for mutual interference with CAT folding. The fact that GFPR1 itself is still somewhat inhibited as the CAT fusion relative to its expression as the free protein, suggests that the wtGFP sequence is still somewhat inhibited by the folding or presence of CAT. The folding of GFPR2, however, was not inhibited at all by the presence of CAT. GFPR2 contains only one mutation in addition to V164A, namely N105S. Thus, this mutation is apparently responsible for the complete elimination of folding interference between GFP and CAT. It is not likely that the combination of mutations in GFPR2 arose by recombination because V164A is apparently indispensable. Rather, the combination probably arose by simple addition of the N105S mutation to V164A. We have confirmed that the N105S mutation by itself is not sufficient to confer a selectable increment in cam resistance on GFP-CAT.

The ability of the surrogate marker fold selection system to select mutations which specifically enhance fusion protein expression in addition to those that enhance independent folding is an added benefit for proteins like GFP, which have important applications as fusion proteins. One question is whether fold selection in the context of fusion proteins could, on occasion, select only mutations which accelerated folding only in the context of the fusion, and did not accelerate folding of the free protein. Such mutations would be highly unlikely because in addition to protecting the protein of interest from interference by the fusion partner, such mutations would also have to in effect convert the fusion partner into a chaperone, for which there is no precedent. Mutations which specifically improve fusion expression, like GFP-N105S, can only be selected in proteins which already fold independently, like GFPR1. In preliminary tests with other fusion partners, GFPR2 has continued to fold independently of the fusion partner, neither inhibiting nor being inhibited by it. For example, as a C-terminal fusion with neomycin phosphotransferase (GFPR2-NPT) both fluorescence and kanamycin resistance were at least as high as those of the free GFPR2 and the free NPT, respectively, whereas both functions were inhibited in the GFPuv-NPT fusion. Since GFPuv was reported to express at 30-fold higher levels than wtGFP in mammalian cells, it may exhibit the same sensitivity to fusion expression in these cells as it does in bacteria. GFPR2 is the subject of US Patent Application 60/160,461.

Example 3

Unstable proteins can be stabilized by peptides selected from random peptide libraries.

Many diseases are caused by unstable proteins, which fail to accumulate in biologically active form in cells or tissues due to one or more mutations which cause a delay in folding or which destabilize the active conformation such that the protein is prone to insoluble aggregation and/or proteolysis. There are two main types of unstable proteinopathies: those which cause disease by forming toxic insoluble aggregates, and those which cause disease by loss of function. The former are represented by amyloidogenic polypeptides such as the amyloid β protein (Aβ), which forms insoluble amyloid fibrils in the brain (Li *et al.*, *J. Leukocyte Biol.* (1999) 66:567-74; Cappai and White, *Int. J. Biochem. Cell Biol.* (1999) 31:885-9). Amyloid deposits can induce chronic inflammation and tissue damage, which are major etiologic components of Alzheimer's disease. There is currently much interest in the development of chemo-therapeutic strategies to counter the progress of amyloidogenesis and resultant tissue degeneration in Alzheimer's and other amyloidogenic proteinopathies. Drugs which could interfere directly with the aggregation of the Aβ protein would be highly desirable. However, no reliable method currently exists for screening chemical libraries for such activities.

We have demonstrated that some unstable proteins can be stabilized by interaction with small peptides obtained from a random sequence library using the "Fold Selector" technology described above. It is expected that such peptides may be used therapeutically, or may be used as leads for the development of therapeutic agents, which can be used to inhibit, or perhaps even reverse the formation of amyloid or other types of toxic insoluble protein deposits. It is further expected that similar peptides could be selected for their ability to stabilize intra-cellular proteins responsible for loss-of-function proteinopathies. Most mutations which lead to loss of physiological function do not disable the active site of a protein per se, but rather they destabilize the active conformation, or they interfere with the folding pathway so that the protein gets trapped in meta-stable intermediates which are prone to aggregation or proteolysis. The reason for this is that the target size for structural mutations in a natural (i.e., highly evolved) protein is typically much greater than the active site(s). Thus a high proportion of inborn errors of metabolism and other genetic disorders are caused by proteins which do not remain properly folded and/or do not fold properly to begin with. Peptides which

stabilize such proteins in vitro could be used to develop cell-penetrating peptido-mimetics which in turn could be used to restore the missing functions by stabilizing the proteins in vivo.

Because amyloidogenic proteins such as the Aβ peptide do not produce screenable or selectable phenotypes, there is no conventional method to select for stabilization of these proteins. However, as we have demonstrated, when unstable proteins are expressed as C-terminal fusions to a protein with a selectable phenotype, the selectable phenotype is destabilized and can be used to select for stabilization of the amyloidogenic protein. Extracellular amyloidogenic proteins may be expressed in the *E. coli* periplasm as C-terminal fusions to TEM-1 β-lactamase with an intervening flexible linker such as $(Gly_4Ser)_3$. TEM-1 β-lactamase is an *E. coli* plasmid-born enzyme which confers resistance to the penicillin class of β-lactam antibiotics (Genbank Accession no. J01749; Sutcliffe, *Proc. Natl. Acad. Sci. USA* (1978) 75:3737-3741). The coding sequence for the 42-amino acid Aβ peptide (Glenner and Wong, *Biochem. Biophys. Res. Commun.* (1984) 3:885-90; Kang *et al.*, *Nature* (1987) 325:733-736) was sub-cloned for expression in the *E. coli* periplasm as a fusion to the N-terminus of β-lactamase as illustrated in Figure 6. When expressed in *E. coli* strain DH5α, the tendency of Aβ to form insoluble aggregates reduced β-lactamase activity such that the cells would not grow on ampicillin concentrations above 200 μg/ml, whereas, when Aβ was removed or replaced with a stable domain of comparable size (c-fos), the host cells grew with quantitative efficiency (i.e., >0.5 colonies per cell) on ampicillin concentrations up to 800 μg/ml. Polyacrylamide gel electrophoresis (PAGE) confirmed that proportionally more β-lactamase partitioned into the insoluble fraction as the Aβ fusion than as either the free enzyme or as the c-fos fusion. Thus, under these expression conditions ampicillin resistance could be used to select for stabilization of the human Aβ protein.

A random peptide-encoding library (RPL) was constructed using synthetic oligonucleotides to encode a chain of 12 randomly selected amino acids at the N-terminus of *E. coli* thioredoxin (*trxA*; Genbank accession no. M54881) with an intervening flexible linker (Gly_4Ser). The expression constructs for this library and Aβ-β-lactamase are illustrated in Figure 6. The expression cassette for the RPL-trx fusion library was assembled in a pUC-based phagemid (Sambrook *et al.*, in Molecular Cloning A Laboratory Manual, 2nd ed., (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 4.17-4.19) to allow rescue as phage, which could then be used to transfect the construct quantitatively into cells

harboring the Aβ-β-lactamase expression construct. At least 10^8 clones of the RPL were rescued as filamentous bacteriophage by infection with helper phage M13K07 (Sambrook *et al.*, in Molecular Cloning A Laboratory Manual, 2nd ed., (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 4.19-4.50). At least 10^9 DH5α cells bearing the Aβ-β-lactamase construct were infected with a 100-fold excess of RPL phage to insure quantitative infection. At least 10^8 independent transfectants were then plated onto solid medium containing 400, 600, and 800 μg/ml ampicillin. 110 colonies were recovered after overnight growth on 400 μg/ml, 19 were recovered from 600 μg/ml, and 4 were recovered from 800 μg/ml. For negative controls, ten clones were selected at random from the unselected RPL/Aβ-β-lactamase co-transformants, and 10^8 cells of each were plated onto 400, 600, and 800 μg/ml ampicillin. After overnight growth no colonies appeared for any clone on any ampicillin concentration. By contrast most of the selected clones replated onto 400 μg/ml ampicillin, 13 replated onto 600 μg/ml, and 2 replated onto 800 μg/ml. Thus, 12-mer peptides were selected which substantially reduced the tendency of Aβ to form insoluble aggregates, as judged by the increased β-lactamase activity. These peptides can be produced synthetically and modified by known methods to increase their stability in vivo. It is expected that this can be accomplished for at least some of the selected peptides without sacrificing their ability to stabilize Aβ against amyloid formation both in vitro and in vivo.

Interestingly, when a similar 12-mer RPL was constrained between the disulfide-forming cysteines in the active site of thioredoxin, and co-expressed with Aβ-β-lactamase fusion, fewer clones were recovered at 400 μg/ml ampicillin, and none were recovered at higher concentrations. It is instructive to consider why such a library might be so much poorer a source of stabilizing peptides than the tethered N-terminal library we used. The thioredoxin active site-constrained RPL and others like it have been widely used to obtain artificial ligands for antibodies, receptors and other proteins of interest (Colas *et al.*, *Nature* (1996) 380:548-550). The peptides in this RPL are constrained into a closed loop on the surface of the protein by the disulfide, and are therefore expected to be somewhat more rigid than the N-terminal peptides in our library. Rigidity is important for high-affinity protein-protein interactions because it minimizes the entropy cost of binding. However, flexibility may be more important for protein stabilization. Conventional protein-protein interactions are surface interactions, whereas stabilizing peptides may need to interact with residues which

become iternalized in the active conformation. Also, the diversity of constructive interactions which can occur between unstable proteins and flexible peptides should be much greater than interactions with the surfaces of rigid proteins. Thus, some of the stabilizing peptides may extend into the interior of the stabilized Aβ protein, and/or may interact with non-contiguous regions of the protein. It is even possible that in cases where instability is due to folding intermediates, and not to a loss of stability of the active conformation, that stabilizing peptides may, in effect, catalyze the folding reaction without remaining structural components of the folded protein.

We also expect that at least some of the Aβ-stabilizing peptides will not require all 12 amino acids for activity, and may be equally active as smaller peptides. We have stabilized other proteins with smaller peptides. For example, we have identified several linear tri-peptides, which when tethered to a carrier protein can stabilize an unstable fragment of β-lactamase. The peptide-stabilized β-lactamase fragment can then complement a second fragment to form active β-lactamase. We believe that the methods described herein can be broadly used to isolate peptides which can stabilize desired proteins, particularly those which do not produce screenable or selectable phenotypes. Such methods are not currently available, and since there are few if any reports in the literature of protein-stabilizing peptides, it is generally not appreciated that unstable proteins can be stabilized at will with appropriate peptides, and possibly small molecules derived therefrom.

The foregoing results suggest a general procedure for selecting protein-stabilizing peptides from unconstrained terminal RPLs, which begins with expressing the unstable protein of choice as a C-terminal fusion with a flexible hydrophilic linker and a selectable marker in the appropriate compartment of *E. coli*, as illustrated in Figure 6. If the protein of choice is a secreted protein, as in the case of the Aβ peptide, β-lactamase may be used as the C-terminal fusion partner to allow periplasmic selection for β-lactam antibiotic resistance. A signal peptide must be encoded at the N-terminus for export of the fusion protein to the bacterial periplasm. It is preferable to use the p15A replicon with chloramphenicol resistance, so that universal RPLs can be constructed in the pUC phagemid with kanamycin resistance. If the protein of choice is cytoplasmic, as in the case of GFP described above, CAT (Genbank accession no. X06403; Rose, *Nucleic Acids Research* (1988) 16:355) may be used as the fusion partner to allow selection for chloramphenicol resistance (Dekeyzer *et al.*, *Protein*

Engineering (1994) 7:125-130; Zelazny and Bibi, *Biochemistry* (1996) 35:10872-10878). In this case it is preferable to use the p15A replicon with ampicillin resistance, so that the universal RPLs in the pUC phagemid with kanamycin resistance can be used.

The first requirement which must be met is that the unstable protein must cause a substantial quantitative reduction in the selectable phenotype. This must be quantified and the minimum stringency must be established for quantitative selection, as was done for the use of ampicillin resistance to select for stabilization of the Aβ protein. One or more universal RPLs may then be quantitatively introduced into cells expressing the fusion of the desired protein with the selector, and the transfectants are then plated onto the minimum concentration of antibiotic which is quantitatively non-permissive for growth of the fusion protein. The number of independent transformants plated should be equivalent to or greater than the size of the RPL, and the minimum non-permissive concentration of antibiotic should allow no colonies to grow from the same number of cells expressing the fusion protein alone. The RPL used was a 12-mer on the N-terminus of thioredoxin, but the RPL may vary in length from 3 to 20 or more residues on either end of the carrier. However, the proportion of unstable peptides in the RPL rapidly increases when the length exceeds ~ 12 amino acids. The carrier may be any stable protein which tolerates terminal fusions well. Selected peptides may be verified by co-expression with the free protein of choice. A substantial increase in the proportion of the protein which partitions into the soluble fraction should be observed in the presence of the selected peptide only and not in the presence of a non-selected peptide.

Example 4

Unstable proteins can be stabilized by tri-peptides selected from random peptide libraries

The most common cause of instability in proteins is the tendency of folding intermediates, which may be accessed either on the folding pathway or by partial unfolding of the folded protein, to form inter-molecular associations between structural elements which normally associate intra-molecularly in the native conformation. Such inter-molecular associations may initiate polymerization reactions which lead to the formation of insoluble aggregates such as amyloid fibrils (Dobson, 1999, *Trends Biochem. Sci.* 24, 329-32). We wished to test the hypothesis that small peptides could be selected from random sequence libraries for their ability to protect unstable proteins from aggregation. To accomplish this we

utilized a fragment complementation system, which we had developed for the enzyme β-lactamase. *E. coli* TEM-1 β-lactamase (Sutcliffe, 1978, *Proc Natl Acad Sc. USA* 75, 3737-41) may be separated into two fragments at E197-L198 which can complement to form active enzyme with the aid of interacting domains such as hetero-dimerizing helixes which are fused to the break-point termini of the fragments (Balint and Her, US Patent Application 60/124,339).

The activity of the β-lactamase fragment complementation system is limited, however, by the stability of the N-terminal fragment, denoted α197. When α197 and the stable C-terminal fragment, ω198 were co-expressed in the *E. coli* periplasm as fusions to the hetero-dimerizing helixes of the c-fos and c-jun subunits of the transcription factor AP-1 (Karin et al., 1997, *Curr Opin Cell Biol* 9, 240-6), only enough β-lactamase activity was produced to confer a plating efficiency of ~1% on 50 μg/ml ampicillin. However, when the fragment fusions were co-expressed with a library of random tri-peptides at the N-terminus of a carrier protein, *E. coli* thioredoxin (*trxA*; Genbank accession no. M54881) with an intervening Gly_4Ser linker, four tri-peptides were independently selected which specifically increased β-lactamase activity to confer 100% plating efficiency on the host cells. These tri-peptides all turned out to have the same sequence, Gly-Arg-Glu (GRE). The GRE tripeptide conferred no resistance to ampicillin in the absence of the interacting helixes, thus it does not stabilize the re-folded fragment complex, but rather it must stabilize the α197 fragment since activity is limited by the amount of soluble α197. Since the GRE tri-peptide had the same stabilizing effect on α197 fragment when a different carrier was used, its activity must be context independent. Thus, an 18 kDa enzyme fragment could be stabilized at least 100-fold by a tri-peptide selected from a random sequence library.

Interestingly, though the GRE tri-peptide could inhibit aggregation of α197, it apparently did not interfere with re-folding of the fragment complex. Since aggregate formation proceeds exponentially, it is exquisitely sensitive to small shifts in the inter-molecular association rate constants (Dobson, 1999). Thus, even weak binding of an excess of the tri-peptide to the interacting surfaces could effectively defeat inter-molecular aggregation. On the other hand, cooperative folding of the fragment complex should readily displace the weakly bound tri-peptides because the effective intra-molecular concentrations of interacting structural elements relative to one another would be much higher than the tri-peptide concentration. In this way the general ability of small peptides to stabilize large proteins without interfering with protein

folding may be understood. We believe this phenomenon is not widely appreciated, and in fact this may be the first demonstration that a functional protein could be deliberately stabilized by something as small as a tri-peptide.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporate by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for obtaining host cells expressing a mutant of a desired protein optimized for expression in said host cells, said method comprising:

expressing a library of mutagenized coding sequences for said desired protein as individual fusion proteins in a multiplicity of said host cells grown under selective conditions, wherein the coding sequence for each fusion protein comprises a member of said library of mutagenized coding sequences operably linked to a coding sequence for a selector protein, expression of which confers ability to grow under said selective conditions on said host cells; and

identifying host cells that express a fusion protein comprising a mutagenized desired protein and a selector protein under selective conditions that are nonpermissive for host cells expressing a fusion protein comprising an unmutagenized desired protein and said selector protein as indicative of host cells expressing a mutant of said desired protein optimized for expression in said host cells.

2. The method according to Claim 1, wherein said protein is heterologous to said host cell.
3. The method according to Claim 1, wherein said selective conditions are exposure to an antibiotic to which said host cells are sensitive in the absence of said selector protein.
4. The method according to Claim 3, wherein said antibiotic is chloramphenicol.
5. The method according to Claim 3, wherein said antibiotic is a β-lactam antibiotic.
6. The method according to Claim 1, wherein said host cells are prokaryotic cells.
7. The method according to Claim 6, wherein said prokaryotic cells are *E. coli*.
8. The method according to Claim 1, wherein said desired protein lacks a selectable phenotype.
9. The method according to Claim 1, wherein said member of said library of mutagenized coding sequences is operably linked to said coding sequence for a selector protein via a peptide linker.
10. The method according to Claim 9, wherein said peptide linker is flexible.
11. The method according to Claim 1, wherein said member of said library of mutagenized coding sequences is operably linked 5' to said coding sequence for a selector protein.
12. A method for obtaining *E. coli* host cells expressing a mutant of a desired protein optimized for expression in said cells, said method comprising: expressing a library of

mutagenized coding sequences for said desired protein as individual fusion proteins in a multiplicity of said host cells grown in the presence of an antibiotic, wherein the coding sequence for each fusion protein comprises a member of said library of mutagenized coding sequences operably linked to a coding sequence for a selector protein, expression of which confers ability to grow in the presence of said antibiotic; and

identifying host cells that express a fusion protein comprising a mutagenized desired protein and a selector protein at a concentration of said antibiotic that is nonpermissive for host cells expressing a fusion protein comprising an unmutagenized desired protein and said selector protein as indicative of host cells expressing a mutant of said desired protein optimized for expression in said host cells.

13. The method according to Claim 12, wherein said antibiotic is chloramphenicol and said selector protein is chloramphenicol acetyl tranferase.

14. The method according to Claim 12, wherein said antibiotic is a β-lactam antibiotic and said selector protein a β-lactamase.

15. A method for obtaining a mutant of a desired protein optimized for expression in a host cell, wherein said mutant maintains at least one or more functional characteristic of interest of a wild type of said desired protein, said method comprising:

isolating a plurality of mutants of said desired protein from host cells obtained according to the method of Claim 1 or Claim 12; and

screening said plurality of mutants for a mutant comprising said functional characteristic of interest.

16. The method according to Claim 15, wherein said functional characteristic of interest of said mutant as compared to a wild type protein is one or more characteristic selected from the group consisting of enzymatic activity, fluorescence, immunogenicity, solubility, pH sensitivity, temperature sensitivity and half-life.

17. The method according to Claim 15, wherein said functional characteristic of interest of said mutant as compared to a wild type protein is one or more characteristic selected from the group consisting of increased solubility, decreased temperature sensitivity and increased half-life.

18. A method for obtaining a mutant of green fluorescent protein having at least one altered characteristic as compared to a wild type green fluorescent protein, said method comprising:

expressing a library of mutagenized coding sequences for said green fluorescent protein as individual fusion proteins in a multiplicity of host cells grown under selective conditions, wherein the coding sequence for each fusion protein comprises a member of said library of mutagenized coding sequences operably linked to a coding sequence for a selector protein, expression of which confers resistance to said selective conditions on said host cells;

identifying host cells that express a fusion protein comprising a mutagenized green fluorescent protein and a selector protein under selective conditions that are nonpermissive for host cells expressing a fusion protein comprising an unmutagenized green fluorescent protein and said selector protein as indicative of host cells expressing a mutant of said green fluorescent protein optimized for expression in said host cells;

isolating a plurality of mutants of said green fluorescent protein from host cells expressing a mutant of said green fluorescent protein optimized for expression in said host cells; and

screening said plurality of mutants for a mutant having at least one altered characteristic whereby a mutant of green fluorescent protein having at least one altered characteristic as compared to a wild type green fluorescent protein is obtained.

19. The method according to Claim 18, wherein said at least one altered characteristic is selected from the group consisting of solubility, fluorescence, stability, and absorption spectrum.

20. The method according to Claim 18, wherein said member of said library of mutagenized coding sequences is operably linked 5' to said coding sequence for a selector protein.

21. A nucleic acid comprising a coding region for a green fluorescent protein, wherein said nucleic acid comprises a mutated sequence at codon 105.

22. The nucleic acid according to Claim 21, wherein said mutated sequence at codon 105 is AGC.

23. The nucleic acid according to Claim 21, further comprising a mutated sequence at codon 165.

24. The nucleic acid according to Claim 23, wherein said mutated sequence at codon 165 is GCT.

25. A nucleic acid comprising a coding region for a green fluorescent protein, where said green fluorescent protein has a serine instead of an asparagine at residue 105.

26. The nucleic acid according to Claim 25, wherein said green fluorescent protein further comprises an alanine instead of a valine at residue 164.

27. A cell comprising a nucleic acid according to Claim 25, wherein the codon for said serine is a codon preferred by said cell.

28. The cell according to Claim 26, wherein said cell is an *E. coli* cell and said codon is AGC.

29. A cell comprising a nucleic acid according to Claim 22, wherein the codons for said alanine and said serine are codons preferred by said cell.

30. The cell according to Claim 29, wherein said cell is an *E. coli* cell and said codons are AGC and GCT respectively.

31. A green fluorescent protein comprising a serine instead of an asparagine at residue 105.

32. The green fluorescent protein according to Claim 31, further comprising a phenylalanine instead of a valine at residue 164.

33. A method for identifying a DNA sequence which encodes a stable polypeptide in a randomly fragmented population of DNA, said method comprising:

expressing members of said randomly fragmented population of DNA as individual fusion expression products in a multiplicity of said host cells grown under selective conditions, wherein the coding sequence for each fusion expression product comprises a member of said randomly fragmented population of DNA operably linked to a coding sequence for a selector protein, expression of which confers resistance to said selective conditions on said host cells;

screening for host cells that express a fusion expression product under selective conditions as indicative of host cells containing a DNA sequence that encodes a stable polypeptide; and

identifying said DNA sequence.

34. The method according to Claim 33, wherein said DNA is cDNA.

35. A method for obtaining peptides that improve at least one of the solubility or functional properties of a free defective protein, said method comprising:

coexpressing in a multiplicity of host cells grown under selective conditions (a) a defective fusion protein comprising said defective protein and a selector protein, expression of which confers resistance to said selective conditions on said host cells and (b) a member of a tethered

random peptide library comprising a linear chain of about 3 to 20 amino acids fused via a flexible linker to a stable carrier;

isolating host cells that express said defective fusion under selective conditions that are not permissive for host cells expressing (a) alone as indicative of host cells expressing a peptide that improves said defective fusion protein whereby host cells containing peptides that improve said defective fusion protein are obtained;

identifying said peptides that improve said defective fusion protein; and

screening said peptides that improve said defective fusion protein for those peptides that improve at least one of the solubility and functional properties of said free defective protein.

36. The method according to Claim 35, wherein said random peptide library is a linear chain of 12 randomly encoded amino acids fused via a flexible linker to the N-terminus of *E. coli* thioredoxin.

37. The method according to Claim 35, wherein said free defective protein is a secreted protein and said selector protein is a secreted protein.

38. A method for obtaining peptides that improve the solubility of a human amyloid β peptide, said method comprising:

coexpressing in a multiplicity of host cells grown under selective conditions (a) a defective fusion protein comprising said human amyloid β peptide and a secreted selector protein, expression of which confers resistance to said selective conditions on said host cells and (b) a member of a tethered random peptide library comprising a linear chain of about 3 to 20 amino acids fused via a flexible linker to a stable carrier;

isolating host cells that express said defective fusion protein under selective conditions that are not permissive for host cells expressing (a) alone as indicative of host cells expressing a peptide that improves said defective fusion protein whereby host cells containing peptides that improve said defective fusion protein are obtained;

identifying said peptides that improve said defective fusion protein; and

screening said peptides that improve said defective fusion protein for those peptides that improve the solubility said human amyloid β peptide.

39. The method according to Claim 38, wherein said linear chain comprises about 3 to 12 amino acids.

40. A complex comprising a peptide identified according to the method of Claim 38 and a human amyloid β peptide.

41. A pharmaceutical composition comprising a peptide identified according to the method of Claim 38.

42. A peptidomimetic of a peptide identified according to the method of Claim 38.

43. A composition comprising the peptidomimetic according to Claim 42.

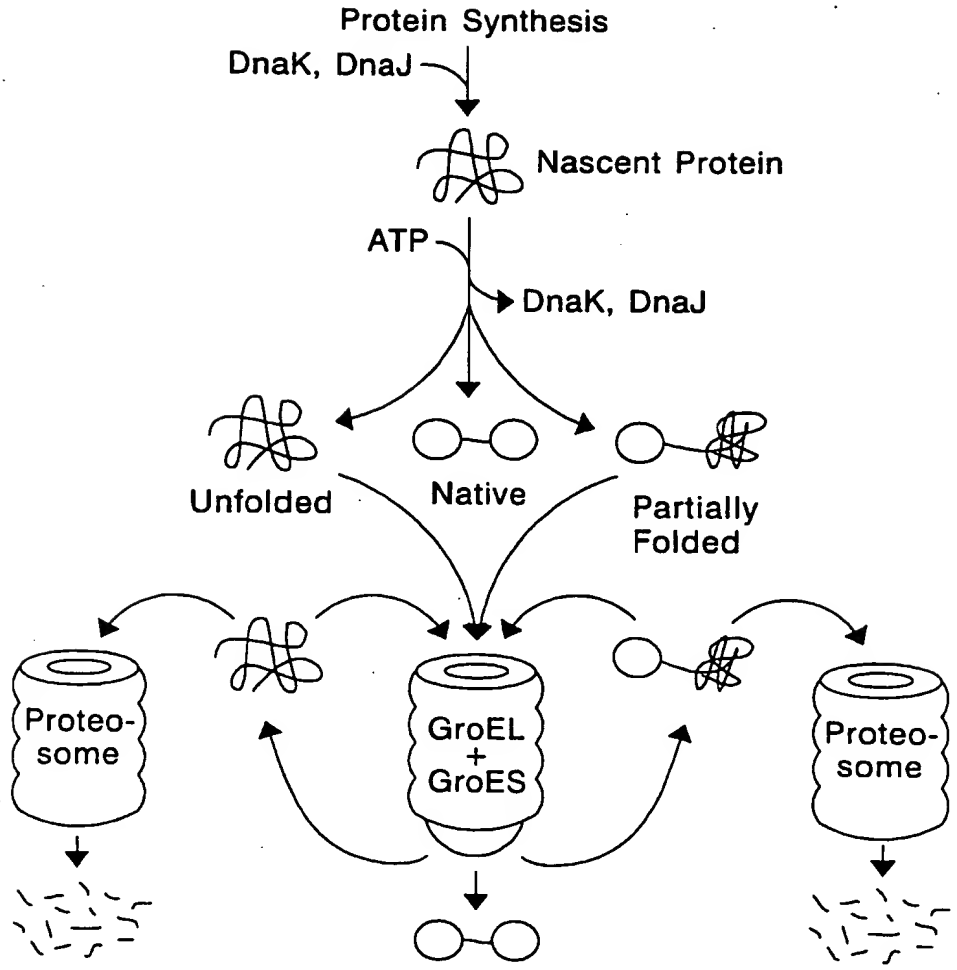

Protein Synthesis
DnaK, DnaJ
Nascent Protein
ATP
DnaK, DnaJ
Unfolded
Native
Partially Folded
Proteo-some
GroEL + GroES
Proteo-some

Figure 1

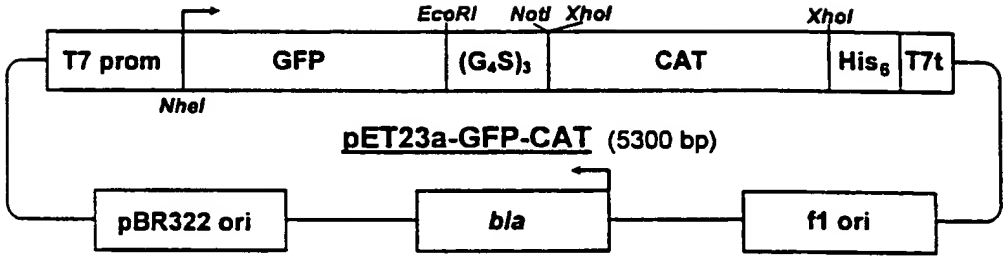

EcoRI
NotI
XhoI
XhoI
T7 prom
GFP
(G4S)3
CAT
His6
T7t
NheI
pET23a-GFP-CAT (5300 bp)
pBR322 ori
bla
f1 ori

Figure 2

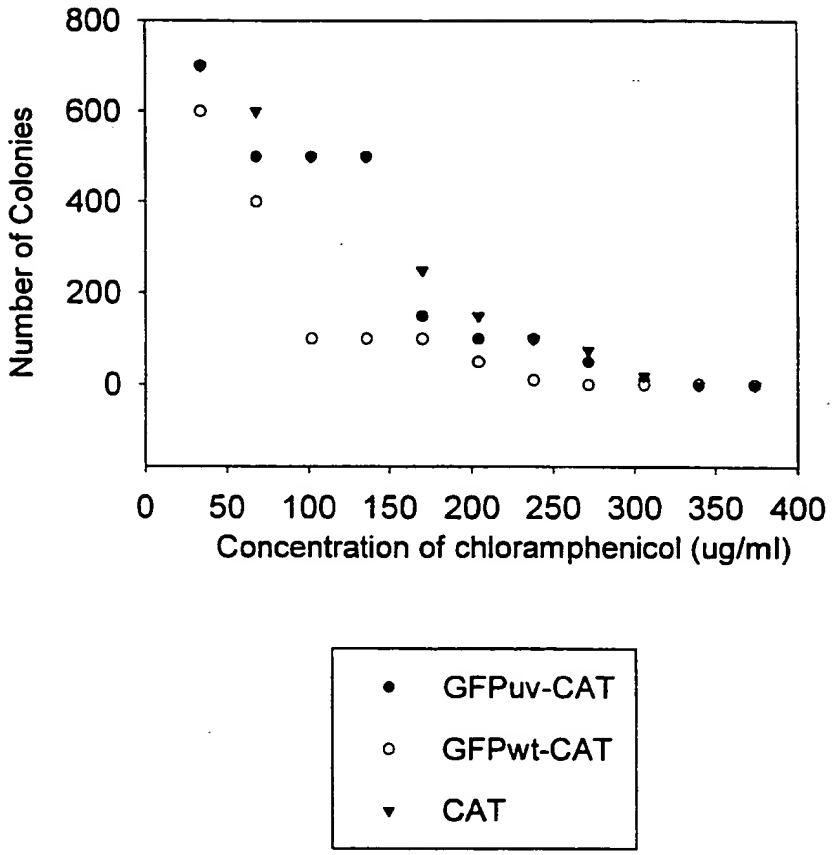

800
600
400
200
0
Number of Colonies
0 50 100 150 200 250 300 350 400
Concentration of chloramphenicol (ug/ml)
GFPuv-CAT
GFPwt-CAT
CAT

Figure 3

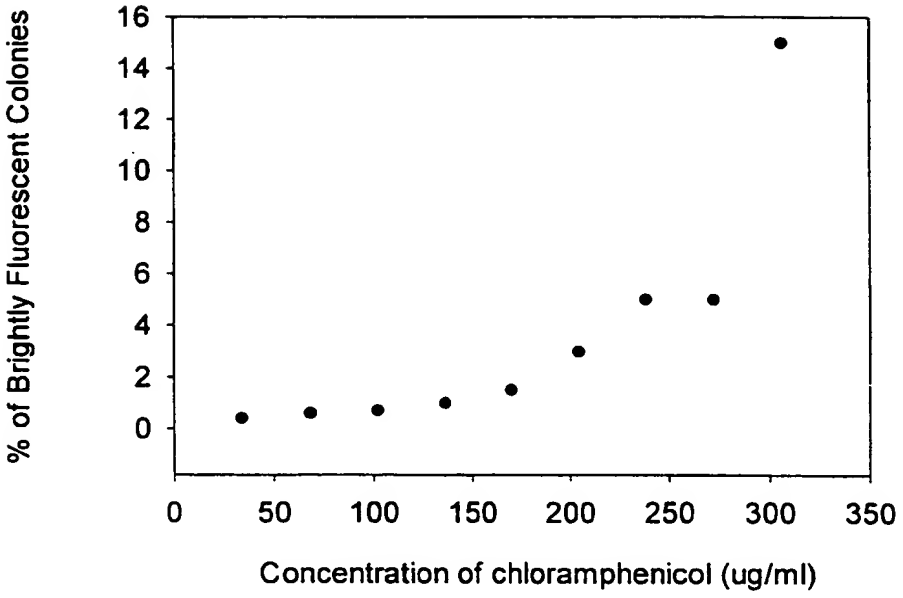

% of Brightly Fluorescent Colonies
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
Concentration of chloramphenicol (ug/ml)

Figure 4

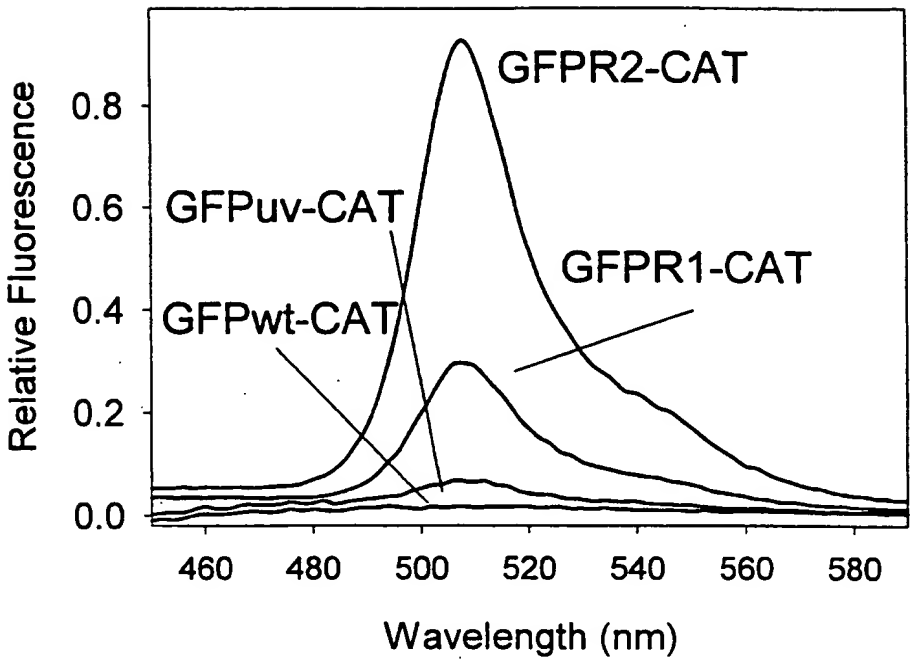

GFPR2-CAT
GFPuv-CAT
GFPR1-CAT
GFPwt-CAT
Relative Fluorescence
0.0
0.2
0.4
0.6
0.8
460
480
500
520
540
560
580
Wavelength (nm)

Figure 5

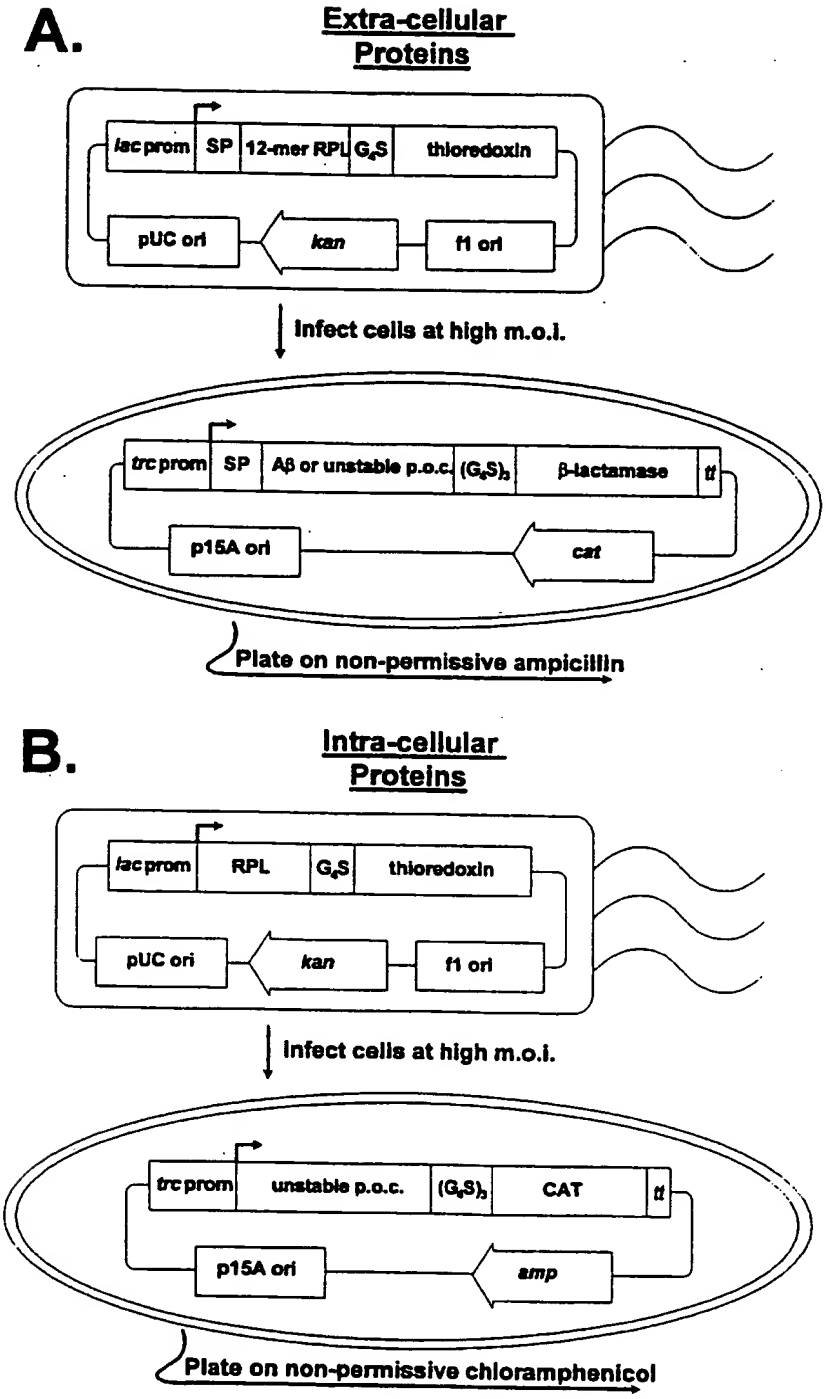

A.
Extra-cellular Proteins
lac prom
SP
12-mer RPL
G4S
thioredoxin
pUC ori
kan
f1 ori
Infect cells at high m.o.i.
trc prom
SP
Aβ or unstable p.o.c.
(G4S)3
β-lactamase
tt
p15A ori
cat
Plate on non-permissive ampicillin
B.
Intra-cellular Proteins
lac prom
RPL
G4S
thioredoxin
pUC ori
kan
f1 ori
Infect cells at high m.o.i.
trc prom
unstable p.o.c.
(G4S)3
CAT
tt
p15A ori
amp
Plate on non-permissive chloramphenicol

Figure 6

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 00/08477

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N15/10 C12N15/62 C07K14/435 C07K14/47 A61K38/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 31266 A (UNIV CALIFORNIA ;WALDO GEOFFREY S (US)) 24 June 1999 (1999-06-24) cited in the application page 1, line 4 - line 10 page 6, line 12 -page 8, line 12 claims 1-27; figures 1,2,4; examples 1-4 ---	1-20,33, 34
X	MAXWELL ET AL.: "A simple in vivo assay for increased protein solubility" PROTEIN SCIENCE, vol. 8, September 1999 (1999-09), pages 1908-1911, XP002146236 abstract page 1908, column 2 -page 1909, column 1; figures 1,2; tables 1,2 --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November 2000	06.12.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir tional Application No
PCT/US 00/08477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 198 08 717 A (SCHMIDT ;SIEBER (DE)) 9 September 1999 (1999-09-09) claims 1-28; figures 1-4	1,2, 6-11, 15-17
X	ROBBEN J ET AL: "Insertional re-activation of a chloramphenicol acetyltransferase misfolding mutant protein" PROTEIN ENGINEERING,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 8, no. 2, 1995, pages 159-165, XP002080426 ISSN: 0269-2139 page 164; figures 1,3; tables 1-3	33,34
X	US 5 962 256 A (MOORE DAVID D ET AL) 5 October 1999 (1999-10-05) column 3, line 5 -column 5, line 51; figure 1	35
X	WO 98 46636 A (AMERICAN HOME PROD) 22 October 1998 (1998-10-22)	35
A	claims 1-24; figures 1,2; example 1	36-39
A	CRAMERI A ET AL: "IMPROVED GREEN FLUORESCENT PROTEIN BY MOLECULAR EVOLUTION USING DNA SHUFFLING" NATURE BIOTECHNOLOGY,US,NATURE PUBLISHING, vol. 14, 14 March 1996 (1996-03-14), pages 315-319, XP000791095 ISSN: 1087-0156 cited in the application page 316, column 2, last paragraph -page 317, column 1, paragraph 1	1-20
A	EP 0 641 861 A (HOFFMANN LA ROCHE) 8 March 1995 (1995-03-08) examples 1-4	35-39
A	EP 0 885 904 A (FRAUNHOFER GES FORSCHUNG) 23 December 1998 (1998-12-23) page 3, line 22 -page 4, line 26; figure 1	35-39
A	FINDEIS M A: "BETA-AMYLOID AGGREGATION INHIBITORS" CURRENT OPINION IN CPNS INVESTIGATIONAL DRUGS,PHARMA PRESS, LONDON,,GB, vol. 1, no. 3, 1999, pages 333-339, XP000933869 ISSN: 1464-844X page 336 -page 337	35-39

-/--

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

	International Application No PCT/US 00/08477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 00 20574 A (RIGEL PHARMACEUTICALS INC) 13 April 2000 (2000-04-13) page 3 -page 4; claims 1-24 -----	1-17,33, 34

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/08477

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 40-43
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1-20,33-43

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 40-43

Claims 40-43 refer to a peptide and peptidomimetic that improve the solubility of beta-amyloid without giving a true technical characterization. Moreover, no such peptides are defined in the application. In consequence, the scope of said claims is ambiguous and vague, and their subject-matter is not sufficiently disclosed and supported (Art.5 and 6 PCT). No search can be carried out for such purely speculative claims whose wording is, in fact, a mere recitation of the results to be achieved.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20

A method for obtaining host cells expressing a mutant of a desired protein optimized for exression in said cells, said method comprising: Expressing a library of mutagenized coding sequences for said protein as fusion proteins to a coding sequence for a selector protein and identifying cells under non-permissive conditions for host cells expressing a fusion protein with an unmutagenized desired protein.
Said method where the selector is an antibiotic resistance marker and the desired protein is green fluorescent protein

2. Claims: 21-32

A nucleic acid comprising a coding region for a green fluorescent protein with a mutated sequence at codon 105, preferentially coding for a serine instead of an asparagine. A cell comprising the above mentioned nucleic acid. A green fluorescent protein comprising a serine instead of an asparagine at residue 105.
The above mentioned nucleic acid additionally comprising a mutated codon 164. A cell comprising said nucleic acid and a green fluorescent protein comprising the additional mutation at codon 164.

3. Claims: 33,34

A method for identifying a DNA sequence which encodes a stable polypeptide in a randomly fragmented population of DNA, preferably cDNA, said method comprising:
Expressing a library of randomly fragmented sequences as fusion proteins to a coding sequence for a selector protein, which can confer resistance to selective conditions to a cell, and identifying cells under selective conditions indicative for host cells expressing a fusion protein comprising a stable polypeptide.

4. Claims: 35-43

A method for obtaining peptides that improve at least one of the solubility or functional properties of a free defective protein, said method comprising:
-coexpressing a) a fusion protein comprising said defective protein and a selector protein and b) a member of a tethered random peptide library fused via a flexible linker to a stable carrier;
-isolating host cells expressing under conditions that are not permissive for host cells expressing (a) alone.
-identifying peptides that improve said defective fusion

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

protein and screening said peptides for those peptides that improve at least one of the solubility and functional properties of the defective protein.
Said method where the defective protein is the human amyloid beta peptide.
A complex comprising a peptide identified according to the above mentioned method and a human amyloid beta peptide. A peptidomimetic of said peptide. A pharmaceutical composition comprising said peptide or said peptidomimetic.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ...tional Application No
PCT/US 00/08477

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9931266	A	24-06-1999	AU	1629199	A	05-07-1999
DE 19808717	A	09-09-1999	NONE			
US 5962256	A	05-10-1999	US	5846711	A	08-12-1998
			US	5866686	A	02-02-1999
			AU	685412	B	22-01-1998
			AU	5589094	A	24-05-1994
			EP	0666926	A	16-08-1995
			JP	8504325	T	14-05-1996
			WO	9410338	A	11-05-1994
WO 9846636	A	22-10-1998	AU	7115698	A	11-11-1998
			BR	9808562	A	23-05-2000
			CN	1268973	T	04-10-2000
			EP	0975753	A	02-02-2000
			NO	995062	A	14-12-1999
EP 0641861	A	08-03-1995	AU	666274	B	01-02-1996
			AU	6612294	A	27-01-1995
			CA	2125467	A	07-01-1995
			CN	1098416	A,B	08-02-1995
			CN	1227846	A	08-09-1999
			JP	11043500	A	16-02-1999
			JP	2947401	B	13-09-1999
			JP	7102000	A	18-04-1995
			NZ	260907	A	25-06-1996
			US	5750374	A	12-05-1998
			ZA	9404686	A	06-01-1995
EP 0885904	A	23-12-1998	DE	19725619	A	24-12-1998
			CA	2240005	A	17-12-1998
			JP	11071393	A	16-03-1999
WO 0020574	A	13-04-2000	AU	1516400	A	26-04-2000